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{Exhibit 31}

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Volume LVII

Bioluminescence and Chemiluminescence

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extremely heat-stable protein, back production of ATP will occur (2 ADP $\overset{MK}{\rightleftharpoons}$ AMP + ATP) following the heat deactivation of coupled Reactions (3) and (4). By maintaining relatively low concentrations of ATP [i.e., ADP after Reaction (1)] and of GK, the effects of MK contamination are eliminated. If sufficiently high levels of ADP are present within the GTP extracts (>200 ng of ADP ml^{-1}), a small amount of light will be produced, after sample injection, owing to MK activity contained within the crude luciferase preparations. The amount of light emitted is less than 1% of the activity resulting from an equimolar concentration of GTP; however, if necessary, this source of interference can be evaluated (and corrected for) by measuring the sum of the concentrations of ATP and ADP within each sample extract[20] and relating these values to the reactivity of standard ADP solutions.

Acknowledgments

The author expresses his appreciation to Dr. O. Holm-Hansen for comments, criticism, and encouragement offered during the course of this research and the preparation of this chapter. Dr. F. Azam and Ms. L. Campbell critically reviewed the original manuscript and offered helpful suggestions for improvement. The methodology described in this report was developed under ERDA contract EY-76-C-03-0010 P.A. 20.

[10] Measurement of the Activity of Cyclic Nucleotide Phosphodiesterases with Firefly Luciferin–Luciferase Coupled Assay Systems

By RICHARD FERTEL and BENJAMIN WEISS

The procedures described below are designed to measure cyclic AMP phosphodiesterase and cyclic GMP phosphodiesterase (EC 3.1.4.17) by reaction sequences using firefly luciferin and luciferase in the final step. These assay systems are simple, sensitive, inexpensive, and reproducible.

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Intracellular concentrations of adenosine 3′,5′-cyclic monophosphate (cyclic AMP) and guanosine 3′,5′-cyclic monophosphate (cyclic GMP) affect a number of biochemical and physiologic processes in the cell.[1-6] Accordingly, certain cell functions may be controlled by altering the cyclic nucleotide concentrations in that cell. One way to influence the concentration of the cyclic nucleotides is by activating or inhibiting cyclic nucleotide phosphodiesterases, which catalyze the hydrolysis of the cyclic nucleotides to their 5′-monophosphate analogs. For this reason, these enzymes have been the subject of a number of investigations,[6] and a variety of methods have been devised to measure their activity.[7-10] We have developed for these enzymes assay procedures based on the quantitative coupling of the product of the phosphodiesterase reaction [either adenosine 5′-monophosphate (5′-AMP) or guanosine 5′-monophosphate (5′-GMP)] to adenosine-5′-triphosphate [ATP].[11,12] The concentration of ATP in the reaction mixture is then determined by means of the firefly luciferin–luciferase reaction.[13]

Principle of the Assay Systems

Cyclic-AMP Phosphodiesterase

In this reaction sequence, the phosphodiesterase converts cyclic AMP to its degradation product, 5′-AMP. The 5′-AMP, in the presence of a very low concentration of ATP, which serves as a phosphate donor, is converted by the enzyme myokinase (EC 2.7.4.3) to adenosine-5′-diphosphate

[1] G. A. Robison, G. G. Nahas, and L. Triner, eds. *Ann. N. Y. Acad. Sci.* **185** (1971).
[2] P. Greengard, R. Paoletti, and G. A. Robison, eds. "Advances in Cyclic Nucleotide Research," Vol. 1, Raven, New York, 1972.
[3] P. Greengard and E. Costa, eds. *Adv. Biochem. Psychopharmacol.* **3** (1970).
[4] N. D. Goldberg, R. F. O'Dea, and M. K. Haddox, *in* "Advances in Cyclic Nucleotide Research" (P. Greengard and G. A. Robison, eds.), Vol. 2, p. 155. Raven, New York, 1973.
[5] B. Weiss, ed. "Cyclic Nucleotides in Disease." Univ. Park Press, Baltimore, Maryland, 1975.
[6] B. Weiss, and R. Fertel, *Adv. Pharmacol. Chemother.* **14**, 189 (1977).
[7] R. W. Butcher and E. W. Sutherland, *J. Biol. Chem.* **237**, 1244 (1962).
[8] W. J. Thompson and M. M. Appleman, *J. Biochem.* **10**, 311 (1971).
[9] C. R. Filburn and J. Karn, *Anal. Biochem.* **52**, 505 (1973).
[10] W. Y. Cheung, *Anal. Biochem.* **28**, 182 (1969).
[11] B. Weiss, R. Lehne, and S. Strada, *Anal. Biochem.* **45**, 222 (1972).
[12] R. Fertel and B. Weiss, *Anal. Biochem.* **59**, 386 (1974).
[13] B. L. Strehler and J. R. Totter, *Arch. Biochem. Biophys.* **40**, 28 (1952).

(ADP). ADP is then converted to ATP in the presence of phosphoenolpyruvate, which serves as a phosphate donor, and the enzyme pyruvate kinase (EC 2.7.1.40), which catalyzes the reaction.

Under the conditions specified below, the 5′-AMP is rapidly and completely converted to ATP, which is then measured using the firefly luciferin–luciferase reaction. The entire sequence is shown below:

$$\text{Cyclic AMP} \xrightarrow{\text{phosphodiesterase}} \text{5′-AMP}$$

$$\text{5′-AMP} + \text{ATP} \xrightleftharpoons{\text{myokinase}} 2\ \text{ADP}$$

$$2\ \text{ADP} + 2\ \text{phosphoenolpyruvate} \xrightleftharpoons{\text{pyruvate kinase}} 2\ \text{ATP} + 2\ \text{pyruvate}$$

$$\text{ATP} + \text{luciferin} + O_2 \xrightarrow{\text{luciferase}} \text{AMP} + PP_i + \text{oxyluciferin} + CO_2 + \text{light}$$

Cyclic-GMP Phosphodiesterase

In this reaction sequence, the 5′-GMP formed as the product of the phosphodiesterase reaction alters the equilibrium of the reaction catalyzed by guanosine-5′-monophosphate kinase (EC 2.7.4.8). This leads to a decrease in ATP that is proportional to the concentration of 5′-GMP formed. The ATP concentration is then determined by reaction with firefly luciferin and luciferase. The reaction sequence is as follows:

$$\text{Cyclic GMP} \xrightarrow{\text{phosphodiesterase}} \text{5′-GMP}$$

$$\text{5′-GMP} + \text{ATP} \xrightleftharpoons[\text{kinase}]{\text{guanosine-5′-monophosphate}} \text{GDP} + \text{ADP}$$

$$\text{ATP} + \text{luciferin} + O_2 \xrightarrow{\text{luciferase}} \text{AMP} + PP_i + \text{oxyluciferin} + CO_2 + \text{light}$$

Reagents Used in the Assay Systems

Reagents for the Cyclic-AMP Phosphodiesterase Assay

Reagent A

Glycylglycine buffer, 150 m*M* pH 8.0
Ammonium acetate, 75 m*M*
Magnesium chloride, 9 m*M*

Calcium chloride, 30 μM
Phosphoenolpyruvate, 0.78 mM
Dithiothreitol, 15 mM
ATP, 3 nM

Reactants in this reagent are all at 3 times their final concentration. The reagent is stable for several months at −4°. On the day of the assay, 3% bovine serum albumin (BSA), myokinase, and pyruvate kinase, which are made fresh or stored for short periods at 4°, are added to make the following reagents:

Reagent B
Reagent A, 1 ml
3% BSA, 10 μl
Myokinase, 1.0 μg
Pyruvate kinase, 0.5 μg

Reagent C
Reagent A, 1 ml
3% BSA, 10 μl
Myokinase, 2.0 μg
Pyruvate kinase, 1.0 μg

Reagents for Cyclic-GMP Phosphodiesterase Assay

Reagent D
Glycylglycine buffer, 150 mM, pH 8.0
Magnesium chloride, 6 mM
Calcium chloride, 30 mM
Dithiothreitol, 15 mM

As described above, the reagent is 3 times the final concentration and is stable for several months if stored at −4°. Guanosine-5′-monophosphate kinase and 3% BSA are either made fresh or stored for short periods at 4°. ATP is either made fresh or stored at −4° for up to 1 year.

On the day of the assay these solutions are added to reagent D to make reagent E as follows:

Reagent E
Reagent D, 1 ml
3% BSA, 10 μl
Guanosine-5′-monophosphate kinase, 20 μg
ATP, 0.1–5 μM, depending on desired assay sensitivity

Reagent for Assay of ATP

Reagent F

Morpholinopropanesulfonic acid (MOPS) buffer, pH 7.8, 10 m*M*
Magnesium sulfate, 10 m*M*
0.5% BSA
15 mg/ml purified luciferin–luciferase

Under certain circumstances it may be desirable to increase the concentration of the luciferin–luciferase mixture, or to add additional luciferase.

Source and Preparation of the Reagents

Myokinase (rabbit muscle), pyruvate kinase (rabbit muscle), and guanosine-5′-monophosphate kinase (hog brain) are obtained from Boehringer Mannheim Biochemicals and stored at 4°. The myokinase and pyruvate kinase are received in a suspension of ammonium sulfate. Before use, the suspensions are centrifuged at 1000 *g* for 10 min, and the resulting precipitates, which contain the enzymes, are dissolved in reagent A to the appropriate concentration. Guanosine-5′-monophosphate kinase, which is in a glycerol solution, is used without further treatment.

A purified luciferin–luciferase mixture is obtained from the instrument division of E.I. DuPont de Nemours & Co. Purified luciferase is obtained from either Sigma Chemical Co. or Calbiochem. All other reagents are obtained from Sigma Chemical Co. or Fisher Scientific.

Purification of Cyclic AMP

Cyclic nucleotides obtained from commercial sources generally contain contaminating nucleotides. Although the concentration of these contaminants is low, they may interfere with the assay. Therefore, the cyclic nucleotides must be purified before use.

Cyclic AMP is purified by precipitating the nucleotide contaminants by the addition of solutions of barium hydroxide and zinc sulfate.[14] To each milliliter of a 100 m*M* solution of cyclic AMP, adjusted to pH 7.5, is added 0.25 ml of 0.25 *M* barium hydroxide and 0.25 ml of 0.25 *M* zinc sulfate. The suspension is mixed and centrifuged, and the resulting supernatant fluid is removed, centrifuged again to remove all traces of the precipitate, and passed through a column (7 × 40 mm) of Dowex 50-X8, 200–400 mesh, hydrogen form. The columns are eluted with water, and 1-ml fractions are

[14] G. Krishna, B. Weiss, and B. B. Brodie, *J. Pharmacol. Exp. Ther.* 163, 379 (1968).

collected. The eluent is monitored by reading the absorbance at 259 nm. The concentration of cyclic AMP is calculated and adjusted to 3 m*M* on the basis of its molar extinction coefficient ($E = 15{,}400$).

This solution of cyclic AMP is stable for several months when stored at −4°.

Purification of Cyclic GMP

The cyclic GMP is purified by alumina adsorption[15] and ion-exchange column chromatography. One milliliter of a 20 m*M* cyclic GMP solution in 50 m*M* Tris HCl, pH 8.0, is placed on a 7 × 25 mm neutral alumina column. The eluent from this milliliter is discarded, and the column is eluted with 50 m*M* Tris HCl, pH 8.0. The initial milliliter of Tris eluent is discarded. The next 3 ml contain the purified cyclic GMP. One-milliliter portions of these fractions from the alumina column are placed on a 7 × 25 mm column of Dowex 50-X8. The eluent from this milliliter is discarded. The cyclic GMP is then eluted from the column with 2-ml fractions of water. The fractions containing the highest concentration of cyclic GMP, as determined from optical density measurement (252 nm), are combined. The concentration of cyclic GMP is calculated from its molar extinction coefficient ($E = 13{,}700$) and adjusted to 1 mM. Cyclic GMP purified in this manner typically contains less than 0.005% 5′-GMP and is stable for several months if stored at −4°.

Assay Procedure

Cyclic AMP Phosphodiesterase

This assay is performed in 3 steps. In the first step, the following components are added to a 6 × 50 mm tube: 25 μl of tissue sample containing the unknown phosphodiesterase activity, 25 μl of reagent B, and 25 μl of cyclic AMP.

The reaction sequence is initiated with the addition of cyclic AMP (concentrations from 0.5 μM to 1 mM can be used). The samples are incubated for various times at 37°, placed in a boiling water bath for 5 min to stop the reaction, and cooled. In the second step, 25 μl of reagent C are added to each tube, and the samples are incubated at 37° for 1 hr. In the final step, the samples are assayed for ATP by adding 10 μl of reagent F to each sample and recording the emitted light.

[15] A. A. White and T. V. Zenser, *Anal. Biochem.* **41**, 372 (1971).

Cyclic GMP Phosphodiesterase

This procedure is also performed in 3 steps. In the first step, the following components are added to a 6 × 50 mm tube: 25 μl of tissue sample, 25 μl of reagent D, 25 μl of cyclic GMP.

The reaction is initiated with the addition of cyclic GMP (concentrations from 0.1 μM to 1 mM can be used). The samples are incubated for various times at 37°, placed in boiling water bath for 5 min, and cooled. In the second step, 25 μl of reagent E are added to each tube, and the samples are reincubated at 37° for 1 hr. In the final step, ATP concentration is determined by the addition of 10 μl of reagent F as described above.

Measurement of Generated Light

The light emission can be quantitated either by scintillation spectrometer or by instruments designed expressly for this purpose, which are available from E. I. Dupont de Nemours, Inc., Aminco-Bowman, Inc., and SAI Technology, Inc.

Results

Standard Curves Obtained with These Assay Procedures

To determine the activity of an unknown sample of phosphodiesterase, assays are run with a standard curve, which consists of varying concentrations of either 5′-AMP (when cyclic-AMP phosphodiesterase is assayed), or of 5′-GMP (when cyclic-GMP phosphodiesterase is assayed). Standards are assayed under incubation conditions identical to those used for the unknown samples. This procedure is designed to account for the presence of interfering enzymes (e.g., nucleotidases) in the sample. In addition, samples of tissue are tested to determine whether they contain nucleotide contaminants, such as ATP, ADP, or 5′-AMP, which can increase the background of the assay. If the sample does contain high concentrations of such nucleotides, the blank can usually be reduced by preincubating the tissue sample to allow nucleotidases in the tissue to convert these nucleotides to the nucleosides, which do not interfere with the assay.

A typical standard curve for 5′-AMP is linear from 10 to 10,000 pmol and is essentially identical to the curve obtained with ATP alone (Fig. 1). This indicates that under the assay conditions all the 5′-AMP is converted to ATP.

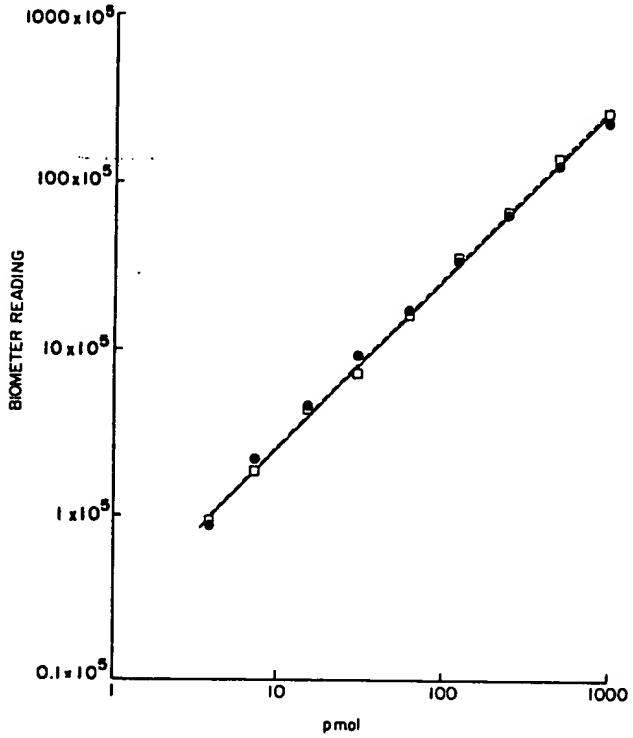


FIG. 1. Conversion of 5′-AMP to ATP. Varying amounts of 5′-AMP (●—●) or ATP (□- - -□) were incubated for 30 min at 37° under conditions described in the test. The light generated by the ATP in the reaction mixture is expressed in arbitrary units. From B. Weiss, R. Lehne, and S. Strada, *Anal. Biochem.* 45, 222 (1972).

The sensitivity of the assay for 5′-GMP is dependent on the initial concentration of ATP in reagent E (Fig. 2). The lower the initial concentration of ATP, the greater the percentage decrease in ATP with a given concentration of 5′-GMP.

Correlation of Phosphodiesterase Activity with Time of Incubation

The optimal incubation time for determination of both cyclic AMP and cyclic GMP phosphodiesterase in a given tissue may vary, but in general, product formation is directly related to incubation time for at least 60 min (Figs. 3A and 3B).

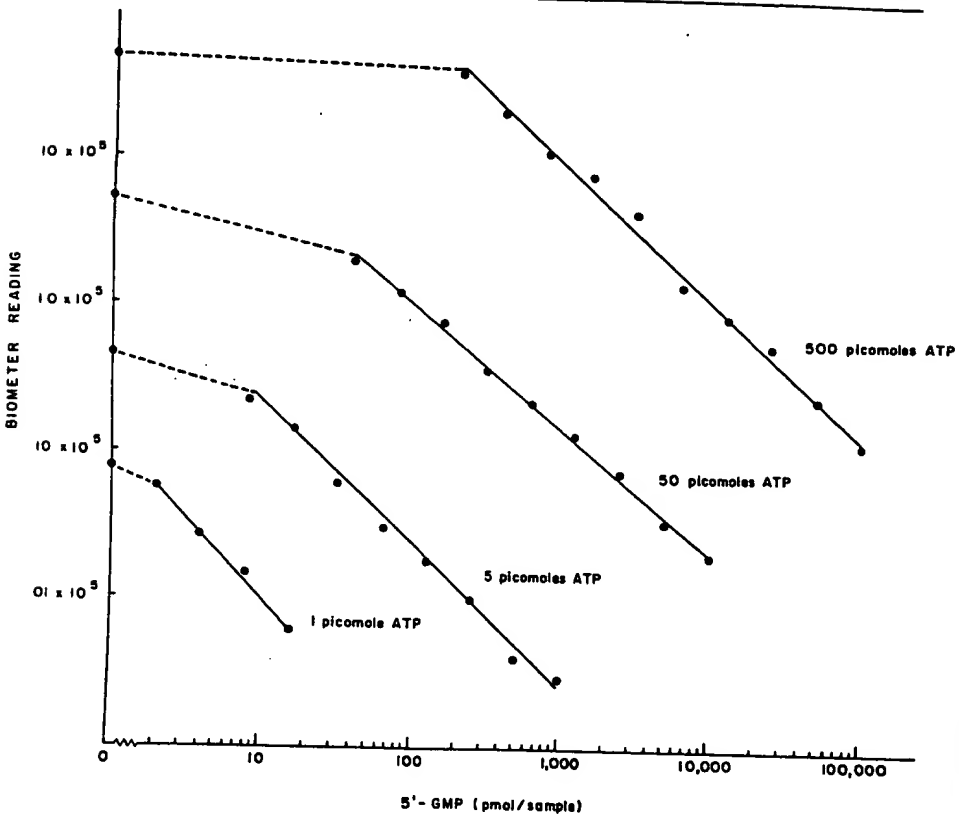
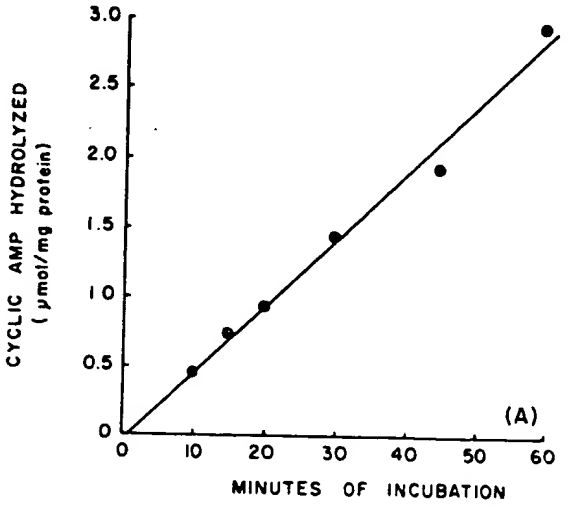
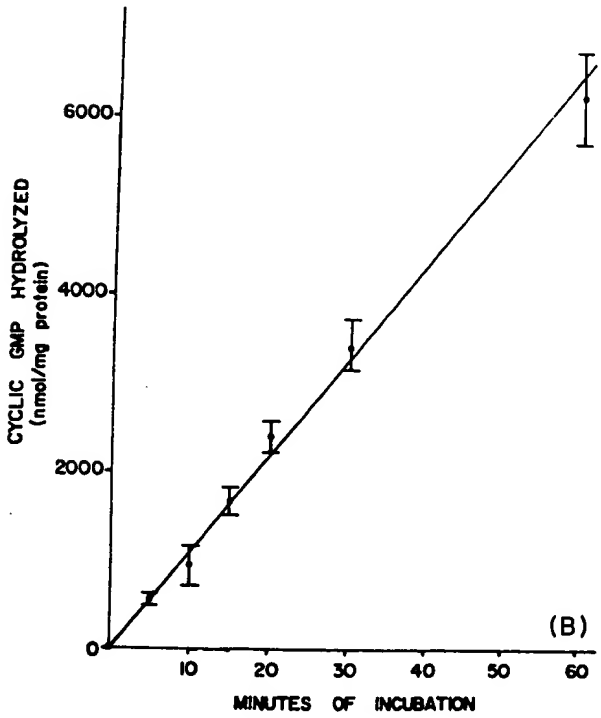


FIG. 2. Sensitivity and linearity of the assay for 5'-GMP. Varying amounts of 5'-GMP and ATP were incubated with reagent E for 60 min at 37°. The amount of ATP that remained is expressed in arbitrary units. Each point represents the mean of five determinations. From R. Fertel and B. Weiss, *Anal. Biochem.* **59**, 386 (1974).

FIG. 3. Hydrolysis of cyclic AMP and cyclic GMP as a function of incubation time. (A) Cyclic AMP phosphodiesterase was measured by incubating a homogenate of rat cerebrum equivalent to 1 μg of tissue with 0.5 m*M* cyclic AMP at 37° for varying times. Each point represents the mean of 3 separate assays. From B. Weiss, R. Lehne, and S. Strada, *Anal. Biochem.* **28**, 182 (1969). (B) Cyclic GMP phosphodiesterase was measured by incubating a 100,000 *g* supernatant fraction of rat cerebral homogenate (equivalent to 0.55 μg of protein) with 0.2 m*M* cyclic GMP at 37° for varying times. Activity was determined as described in the text, using 2 μ*M* ATP in reagent E. Each point represents the mean of 5 determinations. From R. Fertel and B. Weiss, *Anal. Biochem.* **59**, 386 (1974).


CYCLIC AMP HYDROLYZED
(μmol/mg protein)
3.0
2.5
2.0
1.5
1.0
0.5
0
0
10
20
30
40
50
60
MINUTES OF INCUBATION
(A)


CYCLIC GMP HYDROLYZED
(nmol/mg protein)
6000
4000
2000
0
10
20
30
40
50
60
MINUTES OF INCUBATION
(B)

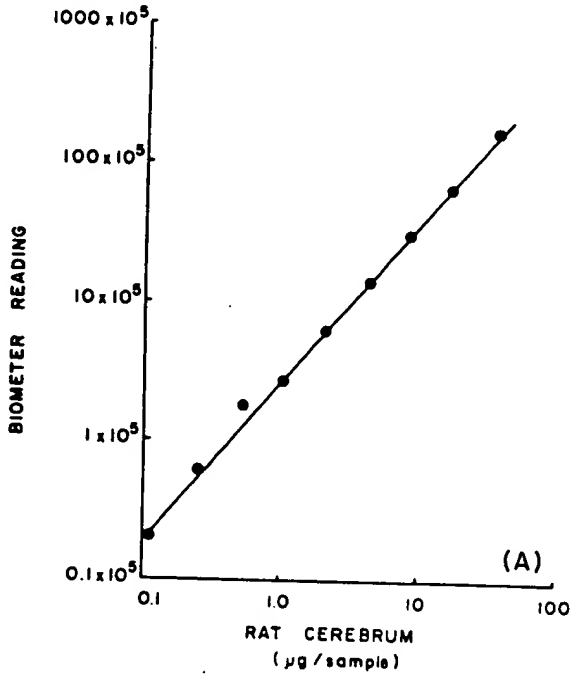


FIG. 4. Hydrolysis of cyclic AMP and cyclic GMP as a function of an increasing concentration of phosphodiesterase. (A) For the cyclic AMP phosphodiesterase, varying concentrations of a homogenate of rat cerebrum were incubated with 0.5 m*M* cyclic AMP for 60 min at 37° as described in the text. From B. Weiss, R. Lehne, and S. Strada, *Anal. Biochem.* **45**, 222 (1972). (B) For cyclic GMP phosphodiesterase, varying concentrations of the 100,000 *g* supernatant fraction of rat cerebral homogenate was incubated with 0.2 m*M* cyclic GMP for 60 min at 37°. Enzymic activity was determined as described in the text, using 2 μM ATP in reagent E. Each point represents the mean of 5 determinations. From R. Fertel and B. Weiss, *Anal Biochem.* **59**, 386 (1974).

Correlation of Phosphodiesterase Activity with the Amount of Tissue Added

Both cyclic AMP and cyclic GMP phosphodiesterase activities increase linearly with increasing amounts of tissue (Fig. 4A, 4B). In both assays, the phosphodiesterase activity of less than 100 ng of cerebral protein can be detected, and there is a linear increase in activity over a wide range of tissue concentrations.

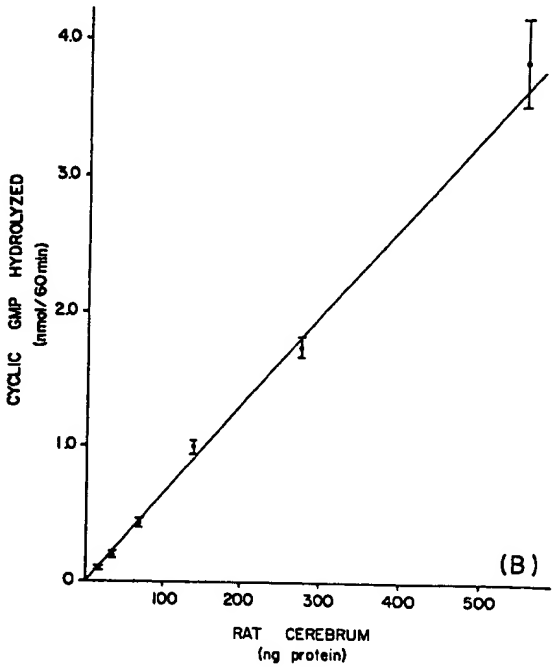


FIG. 4B.

Experimental Results Obtained Using These Assay Procedures

The rapidity and low expense of these procedures, coupled with their high sensitivity, permit the analysis of the large number of samples obtained when the multiple forms of phosphodiesterase are studied. (Several hundred assays per day can easily be performed by one investigator.) For example, analysis of the multiple forms of cyclic-AMP phosphodiesterase of rat cerebrum separated by preparative polyacrylamide gel electrophoresis indicate that there are at least four peaks of cyclic-AMP phosphodiesterase activity (Fig. 5).

These assays also have been used to determine tissue and subcellular distributions of phosphodiesterase isoenzymes, their kinetic parameters, and the response of these enzymes to phosphodiesterase inhibition.[14-20]

[16] R. Fertel and B. Weiss, *Mol. Pharmacol.* **12**, 678 (1976).
[17] B. Weiss, R. Fertel, R. Figlin, and P. Uzunov, *Mol. Pharmacol.* **10**, 615 (1974).
[18] P. Uzunov and B. Weiss, *Biochim. Biophys. Acta* **284**, 220 (1972).
[19] P. Uzunov, H. Shein, and B. Weiss, *Neuropharmacology* **13**, 377 (1974).
[20] P. Uzunov, H. Shein, and B. Weiss, *Science* **180**, 304 (1973).

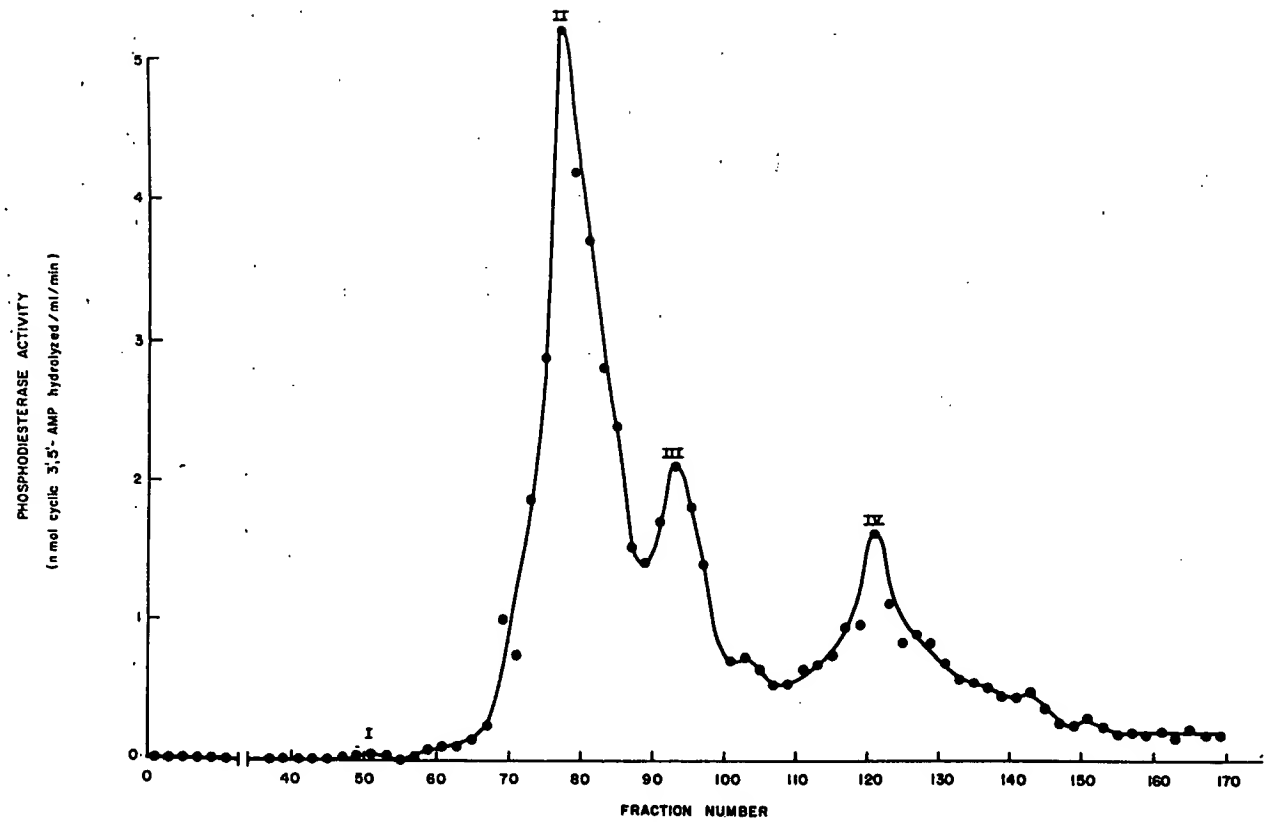


FIG. 5. Rat cerebrum was homogenized in 4 volumes of 0.32 *M* sucrose, sonicated, and centrifuged at 100,000 *g* for 60 min. One milliliter of the supernatant fluid was placed on a preparative polyacrylamide gel electrophoresis apparatus. One-milliliter fractions were eluted from the column, and cyclic AMP phosphodiesterase activity was determined as described in the text, using 0.2 m*M* cyclic AMP as substrate. From B. Weiss, R. Fertel, R. Figlin, and P. Uzunov, *Mol. Pharmacol.* **10**, 615 (1974).